A 01

CODING FORMS FOR SRC INDEXING

| Microfiche No. | | |
|---|---|---|
| OTS0559475 | | |
| New Doc ID | | Old Doc ID |
| 86-980000137 | | |
| Date Produced | Date Received | TSCA Section |
| 09/01/97 | 05/11/98 | 8D |
| Submitting Organization | | |
| INTI ISOCYANATE INST INC | | |
| Contractor | | |
| NATL INST PUBL HLTH, CZECH | | |
| Document Title | | |
| IDENTIFICATION AND ANALYSIS OF 2,4-TOLUENEDIISOCYANATE ADDUCTS WITH GLOBIN AND ALBUMIN, WITH COVER LETTER DATED 5/7/1998 | | |
| Chemical Category | | |
| BENZENE, 2,4-DIISOCYANATE-1-METHYL (584-84-9) | | |

A 02

OFFICE OF TOXIC SUBSTANCES REV. 7/27/82

CODING FORM FOR GLOBAL INDEXING

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Microfiche No. (7) • | | 1 | No. of Pages | | | | 2 |
| Doc I.D. | | 3 | Old Doc I.D. | 8698000137 | | | 4 |
| Case No.(s) | | | | | | | 5 |
| Date Produced (6) | 6 | Date Rec'd (6) | 7 | Conf. Code • | | | 8 |
| | | | | N | | | |
| Check One: ☐ Publication ☐ Internally Generated ☐ Externally Generated | | | | | | | |
| Pub/Journal Name | | | | | | | 9 |
| | | | | | | | 9 |
| Author(s) | | | | | | | 10 |
| Organ. Name | | | | | | | 11 |
| Dept/Div | | | | | | | 12 |
| P.O. Box | 13 | Street No./Name | | | | | 14 |
| City | 15 | State | 16 | Zip | 17 | Country | 18 |
| MID No. (7) | | 19 | D & B NO. (11) | | | | 20 |
| Contractor | | | | | | | 21 |
| Doc Type • • • 8D | | | | | | | 22 |
| Doc Title | | | | | | | 23 |

| Chemical Name (300 per name) | 25 | CAS No. (10) | 24 |
|---|---|---|---|
| | | | |
| | | | |
| | | | |

A 03

6285

INTERNATIONAL ISOCYANATE INSTITUTE, INC.
201 Main Street, Suite 403 • La Crosse, WI 54601 • 608/796-0880 • FAX 608/796-0882

8698000000137

May 7, 1998

TSCA Document Processing Center (TS-790)
Office of Pollution & Toxics
Environmental Protection Agency
401 M Street, SW
201 East Tower
Washington, DC 20460

Contains No CBI

Attn: 8(d) HEALTH & SAFETY STUDY REPORTING RULE
(REPORTING)

Dear Sir or Madam:

We herewith submit a copy of the following recently completed health and safety study.

"IDENTIFICATION OF BINDING SITES FOR DIISOCYANATES IN PROTEINS: A STUDY WITH MODEL AMINO ACIDS AND DIPEPTIDES" and "IDENTIFICATION AND ANALYSIS OF 2,4-TOLUENEDIISOCYANATE ADDUCTS WITH GLOBIN AND ALBUMIN"

| | |
|---|---|
| Name of Chemical Substance: | benzene, 2,4- diisocyanato-1-methyl |
| Common name | 2,4-toluene diisocyanate |
| Chemical Abstracts Service Number: | 584-84-9 |
| Abbreviation: | 2,4-TDI |
| Project Number: | 130-EU-MTX |
| Authors | J. Mraz and S. Bousk |

The International Isocyanate Institute (III) project identification number (11286 and 11287) has been marked on the title page of the reports. Please refer to the III identification number in any communication regarding this study. The enclosed reports do not contain any Confidential Business Information.

This study was sponsored by the International Isocyanate Institute on behalf of the following:

The Dow Chemical Company
ARCO Chemical
Bayer Corporation
BASF Corporation
ICI Americas, Inc.

Very truly yours,

M.J. Blankenship
Managing Director

RECEIVED
OPPT NCIC
98 MAY 21 AM 7:48

A 04

III Project 130

III ref. 11287

# Identification and analysis of 2,4-toluenediisocyanate adducts with globin and albumin

J Mráz

National Institute of Public Health
Prague, Czech Republic



May 1998

Number of pages: 1+19+14

International Isocyanate Institute, Inc.

Project 130-EU-MTX

Final report

Part 2

# Identification and analysis of 2,4-toluenediisocyanate (2,4-TDI) adducts with globin and albumin

Jaroslav Mráz

National Institute of Public Health, Prague, Czech Republic

Collaborators: Š. Bouškova, H. Nohová, E. Gálová, P. Šimek, I. Linhart, J. Šmejkal

September 1997

A 06



Contents:

## 1. Introduction

In the first stage of the EU-130-MTX project, reactivity of proteinogenic amino acids with diisocyanates under physiological conditions have been studied *in vitro* using model compounds. The following nucleophilic groups were recognized as reactive with 2,4-TDI: N-terminal amino acids, cysteine, lysine and histidine residues. These are presumably the reactive sites in protein molecules to which diisocyanates are attached both *in vitro* and *in vivo*.

The aim of the second stage of the project was to assess formation of the amino acid adducts produced at the *in vitro* incubation of 2,4-TDI with native blood proteins globin and albumin. First, standards of the adducts were synthetised and characterized. Then, conjugates of blood proteins with 2,4-TDI were prepared and analyzed using newly developed methods for the individual adduct species.

2,4-TDI contains two chemically unequivalent -NCO groups. As a consequence, three possible adducts may be formed by the reaction of 2,4-TDI with any monofunctional nucleophilic compound in aqueous solutions: two isomeric mono-adducts (in which diisocyanate was bound to the nucleophile via one -NCO group while the second -NCO group was hydrolyzed to amine) and one bis-adduct.

Previously we have synthetized N-acetyl-S-[4-(2-amino)tolylcarbamoyl]cysteine (4-TMA) (Fig. 1), the 2,4-TDI-related monofunctional thiocarbamate capable to transfer the carbamoyl moiety to other nucleophilic sites. In this study, 4-TMA was used to prepare protein conjugates containing exclusively 4-(2-amino)tolylcarbamoyl- out of the three possible (and two isomeric) 2,4-TDI-related adducts. Analysis of the 2,4-TDI- and 4-TMA-related conjugates together facilitated interpretation of the adduct patterns.

Adducts of 2,4-TDI (4-TMA) with the following reactive sites in blood proteins have been considered for further research:

a) N-terminal valine (globin)

b) N-terminal aspartic acid (albumin)

c) $NH_2$-group of lysine residue (globin and albumin)

d) SH- group of cysteine residue (globin and albumin)

e) NH-group of imidazolyl ring of histidine residue (globin and albumin).

## 2. Material and procedures

### Blood proteins

Human full blood and human plasma samples were obtained from the blood transfusion unit.
Pure HSA used was Albumin, human, fraction V, 96-99% (A-1653) from Sigma
Pure BSA used was Albumin, bovine, fraction V, minimum 96% (A-2153) from Sigma

### Protein conjugates.

The following conjugates (at least two batches of each type) were prepared:

| | |
|---|---|
| - globin from human blood incubated with 2,4-TDI, 1 and 10 µmol/ml | HG2,4-TDI(1,10) |
| - globin from human blood incubated with 4-TMA, 1 and 10 µmol/ml | HG4-TMA(1,10) |
| - HSA from human blood incubated with 2,4-TDI, 1 and 10 µmol/ml | HSA(bl)2,4-TDI(1,10) |
| - HSA from human blood incubated with 4-TMA, 1 and 10 µmol/ml | HSA(bl)4-TMA(1,10) |
| - HSA from human plasma incubated with 2,4-TDI, 1 and 10 µmol/ml | HSA(pl)2,4-TDI(1,10) |
| - HSA from human plasma incubated with 4-TMA, 1 and 10 µmol/ml | HSA(pl)4-TMA(1,10) |
| - HSA incubated with 2,4-TDI, 10 and 100 µmol/ml | HSA2,4-TDI(10,100) |
| - HSA incubated with 4-TMA, 10 µmol/ml | HSA4-TMA(10) |
| - BSA incubated with 2,4-TDI, 10 and 100 µmol/ml | BSA2,4-TDI(10,100) |
| " 2,4-TDI in oil, 10 µmol/ml | BSA2,4-TDI/oil(10) |
| - BSA incubated with 4-TMA, 10 µmol/ml | BSA4-TMA(10) |

A 09



**Incubation procedures**

*a) Incubation of blood with 2,4-TDI (10 μmol/ml)*

To 10 ml of full blood (37°C) was added dropwise 0.5 M 2,4-TDI in dioxane (0.2 ml) while the tube with blood was shaken in hand. The sample was then left in shaking w ·er bath (37°C) for 1 h and blood was taken for isolation of globin and albumin.

*b) Incubation of plasma or albumin solution with 2,4-TDI (10 μmol/ml)*

To 10 ml of plasma or 5% solution of albumin (HSA or BSA) in 0.1M phosphate buffer (37°C), 0.5M 2,4-TDI in dioxane (0.2 ml) was added in one portion and the tube was shaken on vortex for 5 min. Then it was left in shaking water bath (37°C) for 1 h. The sample was centrifuged at 3000 rpm for 10 min and clear solution was taken for isolation of albumin.

Alternatively, 2,4-TDI was added to plasma (10 ml) as 0.1M solution in olive oil (1 ml) dropwise on vortex mixer. The sample was shaken on vortex for another 5 min and then left in shaking water bath (37°C) ove .ight.

Note: Incubations with 2,4-TDI, 1 μmol/ml were carried out by the addition of 0.05M 2,4-TDI in dioxane (0.2 ml) to blood, plasma or albumin solution (10 ml).

*c) Incubation of albumin solution with 2,4-TDI, 100 μmol/ml*

1 mmol (146 μl) of pure 2,4-TDI was added dropwise to 10 ml of albumin solution on vortex mixer, shaken for another 5 min and then left in shaking water bath (37°C) overnight.

*d) Incubation of blood, plasma or albumin solution with 4-TMA (10μmol/ml)*

To 10 ml of the above material was added 100 μmol (31.1 mg) of solid 4-TMA and the mixture was left in shaking water bath (37 °C) for 24 h. Globin and albumin were then isolated as described above.

Note: Incubations with 4-TMA, 1 μmol/ml were carried out by the addition of 3.1 mg of solid 4-TMA to blood, plasma or albumin solution (10 ml).

A 10



**Protein isolation procedures**

*a) Isolation of globin from full blood*

Blood (10 ml) was separated to erythrocytes and plasma by centrifugation at 2500 rpm for 15 min. Plasma was removed, erythrocytes were washed twice with saline (50 ml) and hemolyzed with distilled water (2 ml). Globin was precipitated from the hemolyzate by cold 2% hydrochloric acid in acetone (30 ml), washed three times with cold acetone (30 ml), once with cold diethylether (30 ml) and dried in rotary vacuum evaporator Gyrovap at room temperature for 30 min, then at 50°C for 90 min.

*b) Isolation of albumin from plasma*

Albumin was isolated from plasma by fraction precipitation with ammonium sulphate. To plasma (5 ml) was added dropwise saturated solution of ammonium sulphate (7.5 ml) while the tube with plasma was shaken on vortex mixer. Precipitate was spun down at 4000 rpm for 20 min. Supernatant was transferred to another tube and cooled. 1M acetic acid (2 ml) was added to precipitate albumin, which was spun down at 3000 rpm for 10 min. Supernatant was removed and albumin was washed twice in a procedure in which it was dissolved in 5 ml of 1M acetic acid and precipitated by 5 ml of acetone again. Albumin (still containing large amount of ammonium sulphate) was then dried in rotary vacuum evaporator (Gyrovap) at 60°C. Later it was dissolved or suspended in 10 ml water and dialyzed overnight against running water. Washed albumin was freeze dried.

*c) Isolation of albumin from its aqueous solutions*

Albumin was precipitated from its 5% aqueous solution (10 ml) by stepwise addition of cold acetone (25 ml) while the tube was shaken on vortex mixer. Precipitate was spun down, washed twice by 20 ml acetone, and dried in rotary vacuum evaporator (Gyrovap) at 60°C. The material was not washed to remove low molecular weight impurities.

## 3. Adducts with N-terminal valine of globin

Adducts of 2,4-TDI with N-terminal valine of globin have been studied in our laboratory extensively. The obtained results are summarized below.

***Analysis of hydantoins by gas chromatography***

Binding of 2,4-TDI to N-terminal valine via isocyanate groups in position 2-, 4- or both 2- and 4- (which means linkage of two globin chains) produces ureid adducts which are converted to and determined as 3-[2-(4-amino)tolyl]-5-isopropylhydantoin (2-TVH), 3-[4-(2-amino)-tolyl]-5-isopropyl-hydantoin (4-TVH) and 2,4-toluylene-3,3´-bis(5-isopropylhydantoin) (2,4-TDVH) (Fig.1), respectively.

Standards of 2-TVH, 4-TVH and 2,4-TDVH were prepared and characterized. Mass specura are shown in Fig.2.

The procedure for analysis of the above ureids/hydantoins in globin consists of the following steps: isolation of globin from blood (see Protein isolation procedures...); treatment of globin with concentrated hydrochloric-acetic acid (2:1) at 100°C for 8 h; saturation of the acidic mixture with ammonium sulphate and adjustment to pH=7; extraction with ethyl acetate, concentration of the extract; gas chromatographic analysis.

Analysis of globin from human blood incubated with 2,4-TDI revealed presence of all three hydantoins 2-TVH, 4-TVH and 2,4-TDVH. In routine measurements, however, only 2-TVH and 4-TVH were determined (Fig.3); 2,4-TDVH is eluted much later and/or at a higher temperature. Analysis of globin from human blood incubated with 4-TMA revealed presence of 4-TVH.

Level of 4-TVH in globin samples prepared in this study was as follows (μmol/g, average of 2 batches): $HG_{2,4\text{-TDI}(1)}$, 0.33; $HG_{2,4\text{-TDI}(10)}$, 2.45; $HG_{4\text{-TMA}(1)}$, 2.04; $HG_{4\text{-TMA}(10)}$, 18.0; (total N-terminal valine in globin: 64 μmol/g).

The respective ureid adducts were produced also *in vivo* after intraperitoneal administration of 2,4-TDI and 4-TMA to rats. Toxicokinetics of the ureid adduct measured as 4-TVH reflects the rate of removal of erythrocytes from the circulation (life-time of 65 days).

*Analysis of ureids by HPLC*

Total enzymatic hydrolysis of globin containing ureid adducts of 2,4-TDI with N-terminal valine was expected to split the peptidic bond between valine and the neighbouring amino acid (Leu in HG α-chain, His in HG β-chain) but not the ureid bond. Thus, ureids consisting of 2,4-TDI and valine moieties such as 4-(2-amino)tolylcarbamoylvaline (4-TVU) (Fig.1) should be produced.

Standard of 4-TVU was prepared by alkaline treatment of 4-TVH and characterized by ESI-MS (MW=265).

Enzymatic hydrolysis of HG4-TMA(10) with Pronase followed by HPLC analysis (see Adducts with $NH_2$-group of lysine residue) revealed presence of 4-TVU in the hydrolysate (Fig.11). Identity of 4-TVU was confirmed by coelution with the standard in HPLC and by presence of identical ions in their ESI mass spectra [PESI: 288 $(M+Na)^+$; PESI-MS-MS, parent 266: 118; NESI: 264 $(M-H)^+$; NESI-MS-MS, parent 264: 116].

References:

1) Mráz J., Linhart I., Weidenhoffer Z.: Identification of 2,4-toluenediisocyanate (2,4-TDI) adducts with N-terminal valine of globin as potential biomarkers of exposure to 2,4-TDI. Human Exp. Toxicol. 11, 429 (1992).
2) Mráz J., Nohová H., Gálová E., Vaňková H.: In vitro studies on thiocarbamate and ureid adducts of 2,4-toluenediisocyanate (2,4-TDI) in blood proteins (Abstract). Toxicol. Letters, Suppl. 1/78, 58 (1995).
3) Mráz J., Gálová E., Hornychová M.: Biological monitoring of 2,4-toluenediisocyanate (2,4-TDI). Formation and persistence of 4-(2-amino)tolyl adduct with N-terminal valine of hemoglobin in vivo in rats. Abstract in the Proceedings of International Symposium on Biological Monitoring in Occupational and Environmental Health, Espoo, September 1996.

## 4. Adducts with N-terminal aspartic acid of albumin

It was presumed that 2,4-TDI can bind to N-terminal aspartic acid via isocyanate groups in position 2-, 4- or both 2- and 4- (which means linkage of two albumin chains) to produce ureid adducts which can be converted to and determined as 3-[2-(4-amino)tolyl]-5-carboxymethylhydantoin (2-TAH), 3-[4-(2-amino)tolyl]-5-carboxymethylhydantoin (4-TAH) and 2,4-toluylene-3,3´-bis(5-carboxymethylhydantoin) (2,4-TDAH), respectively (Fig.4). These can be analyzed following derivatization by gas chromatography.

Standards of 2-TAH, 4-TAH and the respective methylesters (2-TAH-Me, 4-TAH-Me) were prepared.

Albumin after incubation with 2,4-TDI and 4-TMA was processed as described for globin (Analysis of hydantoins...) except that the acidic mixture was extracted after adjustment to pH=2. The extract was analyzed for 2-TAH and 4-TAH following either methylation (with diazomethane) or silylation (with MTBSTFA).

Further, total enzymatic hydrolysis of albumin containing ureid adducts of 2,4-TDI with N-terminal Asp was expected to split the peptidic bound between Asp and the neighbouring amino acid (Ala in HSA, Thr in BSA) but not the ureid bond. Thus, ureids consisting of 2,4-TDI and Asp moieties such as 4-(2-amino)tolylcarbamoyl aspartic acid (4-TAU) (Fig.1) should be produced.

***Preparation of 2-TAH-Me and 4-TAH-Me***

Aspartic acid dimethylester (50 mmol, 8.38 g) was dissolved in 200 ml dioxane and 2,4-TDI (50 mmol, 8 ml) was added. After mixing for 1 h, clear solution was poured into 0.2M hydrochloric acid (500 ml) with dioxane (200 ml). Solvents were evaporated in rotary vacuum evaporator to dryness, the residuum was dissolved in concentrated hydrochloric-acetic acid (2:1) and heated at 100°C for 4 h. Acids were evaporated to dryness, the residuum was dissolved in MeOH and diazomethane solution in diethylether was added stepwise as long as the reaction was processing (indicated by the evolvment of bubbles of nitrogen). The resulting material was repeatedly chromatographed on silica column with $CHCl_3$-MeOH-acetic acid in various proportions. After evaporation of the solvents, white solids 2-TAH-Me (72 mg) and 4-TAH-Me (232 mg) were obtained. Identity and purity of the compounds was checked by GC-MS, NMR, TLC and HPLC. Mass spectra of 2-TAH-Me and 4-TAH-Me are in Fig.5.

***Preparation of 2-TAH and 4-TAH***

2-TAH and 4-TAH were prepared by treatment of the corresponding methylesters (30 mg) with concentrated HCl (3 ml) at 100°C for 3 h. After evaporation of acid in vacuo, lightly yellow solids were obtained (with the yields of 67 and 92%, respectively).

*Preparation of 2-TAU and 4-TAU*

2-TAU and 4-TAU were prepared by treatment of 2-TAH-Me and 4-TAH-Me (30 mg), respectively, with 2N NaOH (3 ml) at 25°C overnight. Products were purified on silica column with mobile phase $CHCl_3$-MeOH-acetic acid (7:2:1). Yields of the lightly yellow solids were ca 60%.

*Analysis of albumin for ureid adducts as hydantoin methylesters*

Albumin (50 mg) was heated with concentrated hydrochloric-acetic acid (2:1) (5 ml) at 100°C for 8 h. Acidic mixture was saturated with ammonium sulphate, adjusted by 10M NaOH to pH=2 and extracted with ethyl acetate (8 ml). The extract was evaporated to dryness and the residue was dissolved in 50 µl PrOH. To the solution, 200 µl of diazomethane solution in ether was added. Ether was then evaporated and propanolic solution was analyzed by gas chromatography with nitrogen-selective detector or by GC-MS.

*Derivatization of 2-TAH and 4-TAH with MTBSTFA.*

Methanolic solutions of 2-TAH and 4-TAH (100µg) were evaporated in 1.8-ml vials to dryness. To the residues, dried dimethylformamide (100 µl) and N-*tert*-butyldimethylsilyl-N-methyl-trifluoro-acetamide (MTBSTFA) (50 µl) were added, vials were closed with teflon-lined caps and heated in heating block at 110°C for 1 h. Samples were then analyzed by GC-MS (Fig 6).

*Analysis of albumin for ureid adducts as hydantoin TBDMS derivatives*

Albumin was heated with concentrated acid, the acidic mixture was adjusted to pH=2 and extracted with ethyl acetate as described above. The extract was evaporated to small volume, transferred to 1.8-ml vials with teflon-lined caps, and evaporated to dryness. The residue was derivatized as described for 2-TAH and 4-TAH, and the sample was analyzed by GC-MS.

*HPLC analysis of albumin for ureid adducts following total enzymatic hydrolysis*

This was carried out as described below (see Adducts with $NH_2$-group of lysine residue).

*Results and discussion*

Analysis of albumins HSA(pl)2,4-TDI(10), HSA(pl)4-TMA(1), BSA2,4-TDI(10), BSA4-TMA(1,10) by the procedure involving conversion of ureid adducts to hydantoin methylesters did not reveal presence of 2-TAH-Me and 4-TAH-Me in any of the samples (not shown).

Silylation of 2-TAH standard with MTBSTFA provided two products, identified by GC-MS as tris- and tetrakis- TBDMS derivatives. Silylation of 4-TAH standard provided at least four products, identified as one bis-, two tris- and one tetrakis-TBDMS derivatives (Fig.7). Proportion of the products was strongly effected by the reaction conditions; reproducibility of the derivatization was poor. Heating of derivatization mixture at 110°C for 1 h was chosen for analysis of 2-TAH and 4-TAH in albumin.

Analysis of HSA2,4-TDI(10) and BSA2,4-TDI(10) by the procedure involving silylation by MTBSTFA revealed presence of both 2-TAH and 4-TAH while HSA4-TMA(10) and BSA4-TMA(10) produced exclusively 4-TAH (Fig. 6). Unlike with the synthetic standards, single TBDMS derivatives of 2-TAH and 4-TAH were observed in the chromatograms (Fig.6).

Enzymatic hydrolysis of the above albumins with Pronase followed by HPLC analysis did not reveal presence of 2-TAU and 4-TAU in hydrolysate of any of the samples (Figs 9,10). However, because these partially coelute under the given chromatographic conditions with 2-TLU and 4-TLU, respectively, small amounts of 2-TAU and 4-TAU in the hydrolysate may remain undetected.

In conclusion, 2,4-TDI and 4-TMA do produce ureid adducts with N-terminal Asp of albumin. These can be converted to and analyzed as specific hydantoins. The level of these adducts appears to be much lower (we estimate by at least two orders of magnitude) than the level of corresponding adducts with N-terminal valine, providing the incubation procedure was identical. On the other hand, since recovery of the employed analytical procedures was not studied, it cannot be fully excluded that the low yield of the above adducts is also due to low recovery of the analytical procedures used.

## 5. Adducts with $NH_2$-group of lysine residue

It was presumed that 2,4-TDI can bind to lysine residue in proteins via isocyanate groups in position 2-, 4- or both 2- and 4- (which means intra- or intermolecular protein crosslinking) to produce ureid adducts $N_\varepsilon$-[2-(4-amino)tolylcarbamoyl]lysine (2-TLU), $N_\varepsilon$-[4-(2-amino)tolyl-carbamoyl]lysine (4-TLU) and 2,4-toluylene-bis[N-(5-amino-5-carboxypentyl)ureid] (2,4-TDLU), respectively (Fig.8). These can be liberated from the protein chain upon total protein hydrolysis, either acidic or enzymatic.

Standards of 2-TLU, 4-TLU and 2,4-TDLU were prepared.

Globin and albumin after incubation with 2,4-TDI and 4-TMA were subjected to total enzymatic hydrolysis with Pronase and analyzed for the above lysine adducts by HPLC.

*Preparation of 2,4-TDLU*

$N_\alpha$-*tert*-Butyloxycarbonyllysine ($N_\alpha$-*t*BOC-Lys) (20 mmol, 4.92 g) was dissolved in 0.1M phosphate buffer, pH=5 (40 ml) with dioxane (200 ml). 1M 2,4-TDI in dioxane was added in 5-ml portions (total volume: 25 ml) to the solution on mixer until $N_\alpha$-BOC-Lys disappeared. Solvents were evaporated, the solid was dissolved/suspended in chloroform-MeOH and chromatographed on silica column with chloroform-MeOH-acetic acid (8:2:1). Three crude fractions were collected and analyzed by HPLC (column: C18, mobile phase: MeOH-0.2% trifluoroacetic acid 65:35) coupled to mass spectrometer in AP+ ionization mode for components with MW=666 [2,4-TDLU-(*t*BOC)$_2$] and MW=394 (2-TLU-*t*BOC and 4-TLU-*t*BOC). Compounds with MW=394 were not found; the compound with MW=666, which was the major component of the mixture of products, was further purified on silica column and then treated with concentrated hydrochloric acid (room temperature, immediate reaction) to remove the protective *t*BOC groups. Acid was then evaporated in vacuo. Analysis of the product by MS have shown MW=466 (2,4-TDLU) (Tab.1). Identification of 2,4-TDLU was further supported by alkaline hydrolysis (0.3M NaOH, 100°C, 6 h), which produced 2,4-toluene-diamine (2,4-TDA) and Lys, measured as TBDMS derivatives by GC-MS. An attempt to measure TBDMS derivative of 2,4-TDLU by GC-MS was not successful.

*Preparation of 2-TLU and 4-TLU*

Reaction of $N_\alpha$-*t*BOC-Lys with 2,4-TDI in aqueous media produced preferentially bis-ureid adduct even when the reaction conditions (ratio of the components, pH) were varied with the aim to obtain the mono-ureids. In an alternative procedure developed here, 2,4-TDI was first reacted with equimolar amount of alcohol to block half of the -NCO groups (by producing carbamate groups). The reaction mixture containing two isomeric monoisocyanate-mono-carbamate derivatives was then incubated with $N_\alpha$-*t*BOC-Lys to produce the required mono-ureid adducts, and both carbamate and *t*BOC groups were removed by acid hydrolysis.

*Procedure.* Dioxane (50 ml), 2,4-TDI (20 mmol, 3.48 g) and n-PrOH (20 mmol, 1.5 ml) were heated under reflux (100°) for 3 h to produce a reaction mixture containing isocyanatopropyl-carbamates. *t*BOC-Lys (10 mmol, 2.46 g) was dissolved in 10 ml water and pH of the solution was adjusted to 11. Then, the above reaction mixture (30 ml) was added and the solution was mixed at room temperature overnight. Solvents were evaporated, 10 ml of concentrated hydro-chloric acid was added, the mixture was heated at 100°C for 6 h, and acid was evaporated. The resulting material was repeatedly chromatographed on silica column and preparative TLC plates with mobile phase chloroform-MeOH-acetic acid (in various proportions) to obtain yellow solids, ca. 25 mg of each. Molecular weight of 2-TLU and 4-TLU was confirmed by MS (MW=294) (Tab.1). Further, alkaline hydrolysis (0.3M NaOH, 100°C, 6 h) of both compounds produced 2,4-TDA and Lys, identified as TBDMS derivatives by GC-MS. An attempt to measure TBDMS derivatives of 2-TLU and 4-TLU by GC-MS was not successful. Isomerism of the two TLU isomers was assigned indirectly (see Results).

***Analysis of adducts in protein conjugates***

Albumin samples (2 mg) were dissolved in 0.1M phosphate buffer, pH=7.4 (0.5 ml). Globin (2 mg) was dissolved in water (0.25 ml) followed by addition of buffer (0.25 ml). Pronase$^R$ protease from Streptomyces griseus (Calbiochem, cat. Nr. 53702) (50µl of 1% suspension in buffer) was used as a proteolytic enzyme. (For preparative purposes, the amounts were: protein, 20 mg; buffer, 1 ml; enzyme, 5 mg.) The incubation was carried out in shaking water bath (37°C) for 24 h. After incubation, samples were filtered through 0.45µm Durapore filter in Ultrafree filtration units (Millipore) and 20 µl of the filtrate was analyzed by HPLC. The column used was 150x3.9 mm stainless steel cartridge column Nova Pak C18 (Waters) with precolumn filter. Mobile phase was MeOH-0.1% trifluoroacetic acid with gradient elution as

follows: MeOH 5% held for 5 min, then linear increase to 40% during 20 min, held at 40% for another 4 min. Multiwavelength UV detector Waters 490 was set at 250 nm and at absorbance ratio 250/240 nm.

HPLC eluent containing components of interest was collected, concentrated and reanalyzed for purity. The solutions were then analyzed on LCQ LC-MS system (Finnigan) in PESI, PESI-MS-MS, NESI and NESI-MS-MS modes. Samples were introduced to the detector through direct inlet.

***Results and discussion***

Protein conjugates obtained in this study (see Material and Procedures) were subjected to total acidic hydrolysis (6N HCl, 110°C, 24 h) and to total enzymatic hydrolysis with Pronase. The hydrolysates were analyzed by HPLC. Analysis of the acidic hydrolysates have not shown significant differences between control proteins and proteins incubated with 2,4-TDI or 4-TMA with the exception of peak of 2,4-TDA present in chromatograms of most of the protein conjugates (not shown). On the other hand, HPLC analysis of enzymatic hydrolysates of albumins BSA$_{2,4\text{-TDI}(10,100)}$ and HSA$_{2,4\text{-TDI}(10,100)}$ have shown numerous peaks when compared to hydrolysates of control albumins (Figs 9,12). Three of these peaks coeluted with the synthetic lysine adducts, namely, 10/2 and 10/3 with the two TLU isomers and 10/4 with 2,4-TDLU. Components 10/2, 10/3 and 10/4 were collected with the HPLC eluent and characterized by mass spectra in ESI mode. Identical major ions were observed in spectra of the above compounds and the lysine adduct standards (Tab.1). Hydrolysates of the corresponding BSA and HSA conjugates provided very similar chromatograms (Fig.12). Interestingly, the amounts of adducts in albumins incubated with 2,4-TDI doses of 10 and 100 μmol/ml was not significantly different (Fig.12).

2-TLU, 4-TLU and 2,4-TDLU standards were subjected to conditions used in total acidic hydrolysis of proteins (6N HCl, 110°C, 24 h). HPLC analysis have shown that the standards decayed almost completely, producing 2,4-TDA. This is consistent with the failure to detect lysine derivatives in acidic hydrolysates of protein conjugates.

Analysis of hydrolysates of albumins BSA$_{4\text{-TMA}(10)}$ and HSA$_{4\text{-TMA}(10)}$ (Fig.10) revealed peaks some of which were identical with those from albumins incubated with 2,4-TDI. Namely, component 12/1 was identical with 10/3 and 12/4 with 10/5, as assessed by coelution in HPLC and their mass spectra. Moreover, comparison of chromatograms of hydrolyzed

proteins incubated with 4-TMA and with 2,4-TDI allowed assignment of isomerism to the two TLU isomers. Since 4-TMA can only produce 4-TLU, it is obvious that coeluting compounds 12/1 and 10/3 are 4-TLU while 10/2, which is present in albumin incubated with 2,4-TDI but not with 4-TMA, is 2-TLU.

Analysis of hydrolysate of globin $HG_{4\text{-TMA}\ (10)}$ have shown several peaks in addition to those present in control (Fig.11). On the basis of chromatographic coelution and identity of ESI-MS spectra, component 8/1 was identified as 4-TLU whereas the dominant peak 8/4 is 4-TVU (Tab.1). Very small peaks of 4-TLU and 4-TVU were detected also in the chromatogram of hydrolysate of $HG_{2,4\text{-TDI}\ (10)}$ (not shown).

Besides the compounds identified by comparison with the synthetic standards, some other components of the protein hydrolysates were characterized solely by their ESI-MS spectra. These are: 10/5 (MW=442), 10/6 (MW=442), 10/7 (MW=614), 12/2 (MW=336), 12/4 (coeluting with 10/5, MW=442), 8/2 (MW=824), 8/3 (MW=365). An attempt was made to estimate structure of some of these products from the knowledge of their masses. Since spectra of compounds 10/5, 10/6 and 10/7 have some ions in common (Tab.1) it is not unlikely that the relationship between them is analogical to that in compounds 2-TLU, 4-TLU and 2,4-TDLU, namely, 10/5 and 10/6 are isomeric mono-adducts while 10/7 is a related bis-product. Further, some ions in MS spectra of these compounds are in common with the lysine adducts (m/z=293 in NESI-MS-MS). Therefore, we are suggesting that the compounds with MW=442 consist of one Lys and two 2,4-TDI molecules, linked with two ureid bonds, whereas the compound with MW=614 consists of two Lys and two 2,4-TDI molecules, linked with three ureid bonds. Considering the possible formation of these adducts, Lys can only be bound via ε-amino group while cross-linking of 2,4-TDI molecules with ureid link is a major reaction of 2,4-TDI in aqueous medium. Putative formulae of the compounds 10/5 (=12/4), 10/6 and 10/7 are shown in Fig. 14. Comparison of the number of possible positional isomers (4 for MW=442, 4 for MW=614) with the number of observed peaks suggests that some of the isomers are only minor and/or they coelute in HPLC. Presence of one of these products in the hydrolysate of 4-TMA-derived protein adduct (12/4) can be accounted for by a mechanism in which free amino group of the aminotolylcarbamoyl moiety which is already bound to Lys is further carbamoylated by another molecule of 4-TMA.

In conclusion, 2,4-TDI and 4-TMA do produce ureid adducts with lysine residues in proteins. The individual adduct species can be analyzed by HPLC following enzymatic protein

hydrolysis. Whereas lysine adducts appear to be the most abundant adducts of 2,4-TDI and 4-TMA in both human and bovine serum albumin, formation of lysine adducts in globin occurs to much lesser extent. On the other hand, binding to N-terminal amino acid proceeds extensively in globin but to minor extent in albumin.

**Tab.1 Mass spectra of 2,4-TDI adducts with amino acids.**

| Compound (calculated MW) | Characteristic ions (m/z) [a,b] | | | |
|---|---|---|---|---|
| | PESI [b] | PESI-MS-MS | NESI [c] | NESI-MS-MS |
| **2-TLU** (294) | 295*, 317 | 123, 173 | 293*, 329 | 145 |
| 10/2 | 295 | | 293* | 145 |
| **4-TLU** (294) | 295, 317 | | | |
| 8/1 | 295* | 123, 173 | 329 | |
| 10/3 | 295* | 123, 173 | 293* | 145 |
| 12/1 | 295*, 317 | 173 | 293*, 329 | 145 |
| **2,4-TDLU** (466) | 295, 467* | 295, 321 | 465* | 319 |
| 10/4 | 467*, 489 | 295, 321 | 465 | |
| **4-TVU** (265) | 266*, 288 | 118 | 264* | 116 |
| 8/4 | 266*, 288 | 118 | 264*, 116 | 116 |
| 10/5 | | | 441*, 379 | 293 |
| 12/4 | 443*, 465 | 271 | 441*, 379 | 293, 319 |
| 10/6 | 443, 465 | | 441*, 379 | 293, 319 |
| 10/7 | 615*, 637 | 295, 443 | 613*, 193 | 467, 319 |

[a] not always the most abundant peaks

[b] technique PESI produces $(M+1)^+$ and $(M+23)^+$ ions, technique NESI produces $(M-1)^+$ ions

[c] ions marked by asterisk were used as doughter ions in the MS-MS technique

## 6. Adducts with SH- group of cysteine residue

It is presumed that 2,4-TDI can bind to cysteine residue in proteins via isocyanate groups in position 2-, 4- or both 2- and 4- to produce thiocarbamate adducts S-[2-(4-amino)tolylcarbamoyl]cysteine, S-[4-(2-amino)tolylcarbamoyl]cysteine and 2,4-toluylene-bis[S-(2-amino-2-carboxyethyl)thiocarbamate]. Theoretically, these may be analyzed using two different approaches:

i) measurement of the individual thiocarbamate species by HPLC after total hydrolysis of the protein

ii) measurement of free 2,4-toluenediamine (2,4-TDA) released by selective splitting of thiocarbamate bonds (sum of thiocarbamates and perhaps other labile adducts)

However, on the basis of our previous experience no attempt was made to synthetize the above thiocarbamate adduct species and to detect them in proteins after incubation with 2,4-TDI and 4-TMA. The reason for this is as follows:

a) N-aryl thiocarbamates are unstable under physiological conditions. Incubation of blood with 2,4-TDI-related mercapturic acids (i.e., analogues of the above thiocarbamates, N-acetylated in the cysteine moiety) such as 4-TMA resulted in decay of these compounds (half-time of 1-2 h), paralleled by carbamoylation producing more stable adducts.

b) analysis of globin, albumin or plasma after incubation with 2,4-TDI or 4-TMA failed to detect 2,4-TDA bound in such a form which would be reasonably stable under physiological conditions but labile under mildly alkaline conditions.

Reference:

Mráz J., Nohová H., Gálová E., Vaňková H.: In vitro studies on thiocarbamate and ureid adducts of 2,4-toluenediisocyanate (2,4-TDI) in blood proteins (Abstract). Toxicol. Letters, Suppl. 1/78, 58 (1995).

## 7. Adducts with NH-group of imidazolyl ring of histidine residue

An attempt was made to synthetize ureid adducts of 2,4-TDI with histidine residue. $N_{\alpha}$-BOC-His was reacted with 2,4-TDI and with monoisocyanate-monopropylcarbamates derived from 2,4-TDI (as described for preparation of 2-TLU and 4-TLU) until it disappeared from the solution. However, subsequent analysis of the reaction mixture, either before or after treatment with hot concentrated hydrochloric acid, did not indicate presence of products containing both 2,4-TDI and histidine moieties. Instead, abundant free histidine was found in the reaction mixture. Thus, the required products were not prepared.

HPLC analysis of enzymatic hydrolysates of globin and albumin incubated with 2,4-TDI and 4-TMA, followed by off-line ESI-MS analysis of major peaks, did not reveal presence of products containing both 2,4-TDI and histidine moieties.

B 09



## 8. Conclusions

The present report is the first systematic study on the adducts of a diisocyanate (2,4-TDI) with blood proteins globin and albumin. The investigations included preparation of synthetic standards, identification of the individual adduct species in protein conjugates, and development of the provisional analytical procedures. The results are summarized as follows:

a) 2,4-TDI binds to N-terminal valine of globin to produce ureid adducts which can be converted to and determined as specific hydantoins (2-TVH, 4-TVH, 2,4-TDVH). These are suitable biomarkers of the exposure to 2,4-TDI *in vivo*. Routine procedure for gas chromatographic determination of 4-TVH (2-TVH) is available. Highly sensitive analytical method applicable for the purposes of biomonitoring has to be developed.

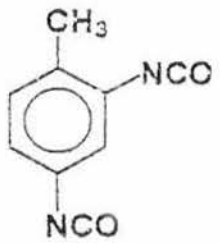
b) 2,4-TDI binds to N-terminal aspartic acid of albumin to produce ureid adducts which can be converted to and detected as specific hydantoins. Binding to this site occurs to very minor extent.

c) 2,4-TDI binds extensively to lysine residues of albumin to produce ureid adducts which can be liberated from the protein by enzymatic hydrolysis and analyzed by HPLC. This procedure is suitable for characterization of 2,4-TDI-albumine conjugates prepared *in vitro*. Binding of 2,4-TDI to lysine residues of globin also occurs, yet to minor extent.

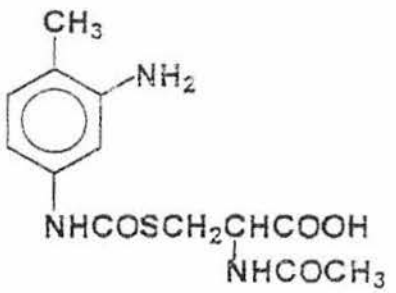
d) 2,4-TDI is very unlikely to produce stable adducts with cysteine and histidine residues of globin and albumin.

e) Enzymatic hydrolysis of protein conjugates with Pronase followed by HPLC is a suitable non-selective procedure for analysis of individual xenobiotic adduct species.

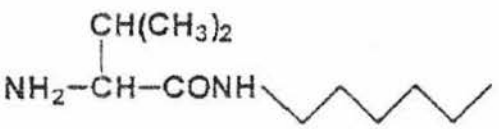
## 9. Figures



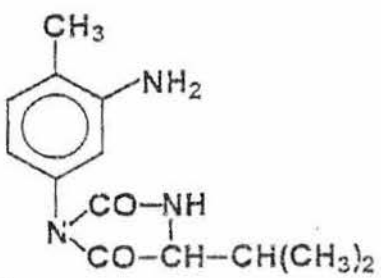
2,4-TDI



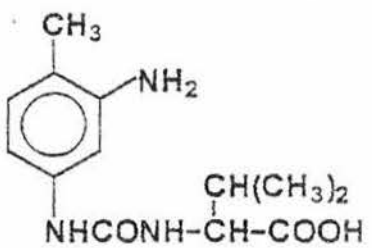
4-TMA



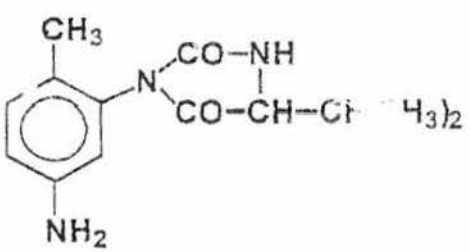
N-terminal Val of globin



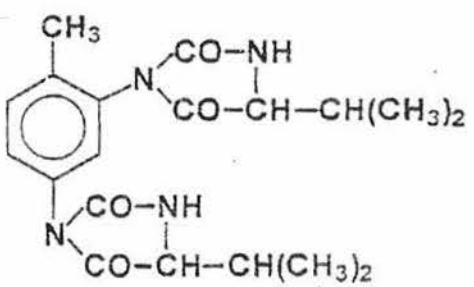
4-TVH



4-TVU



2-TVH



2,4-TDVH

Fig. 1 Adducts of 2,4-TDI with N-terminal valine

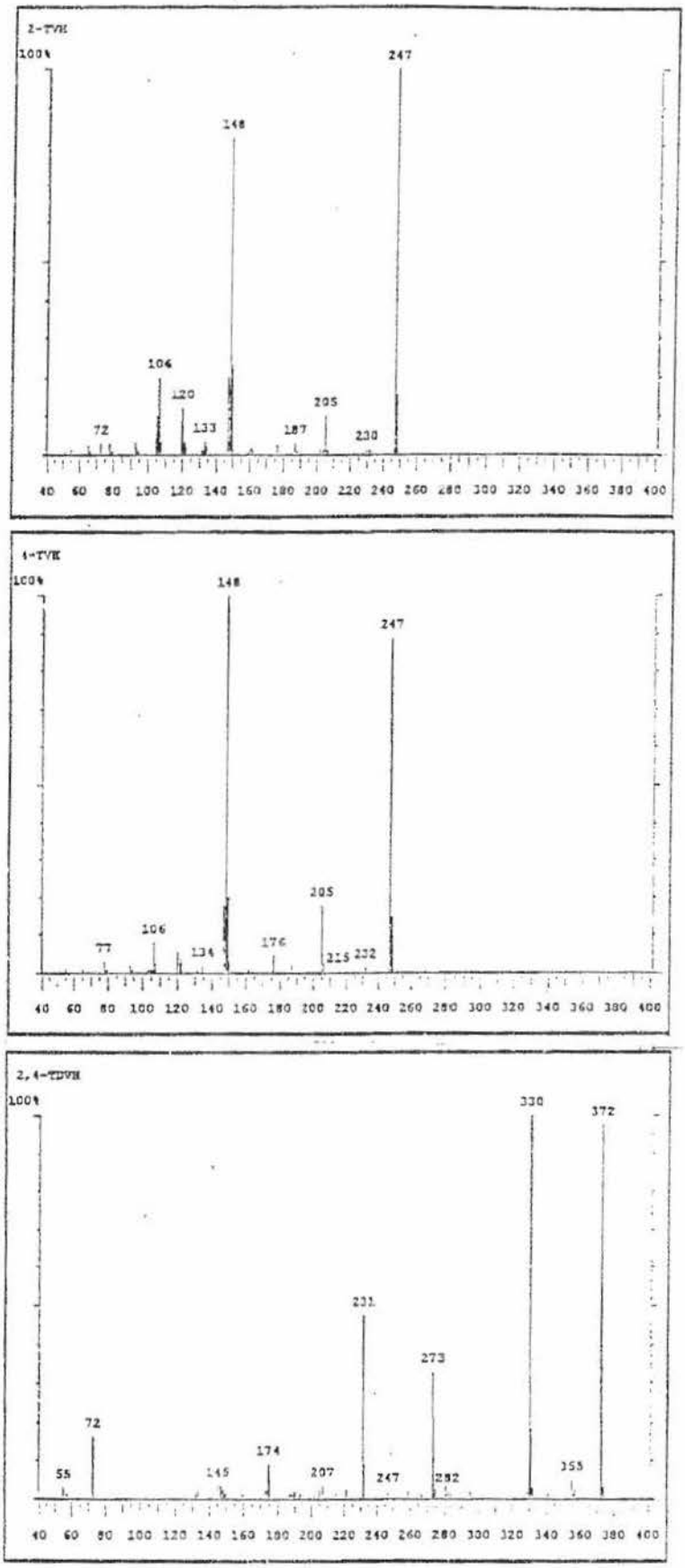



Fig. 2 Mass spectra of 2-TVH, 4-TVH and 2,4-TDVH

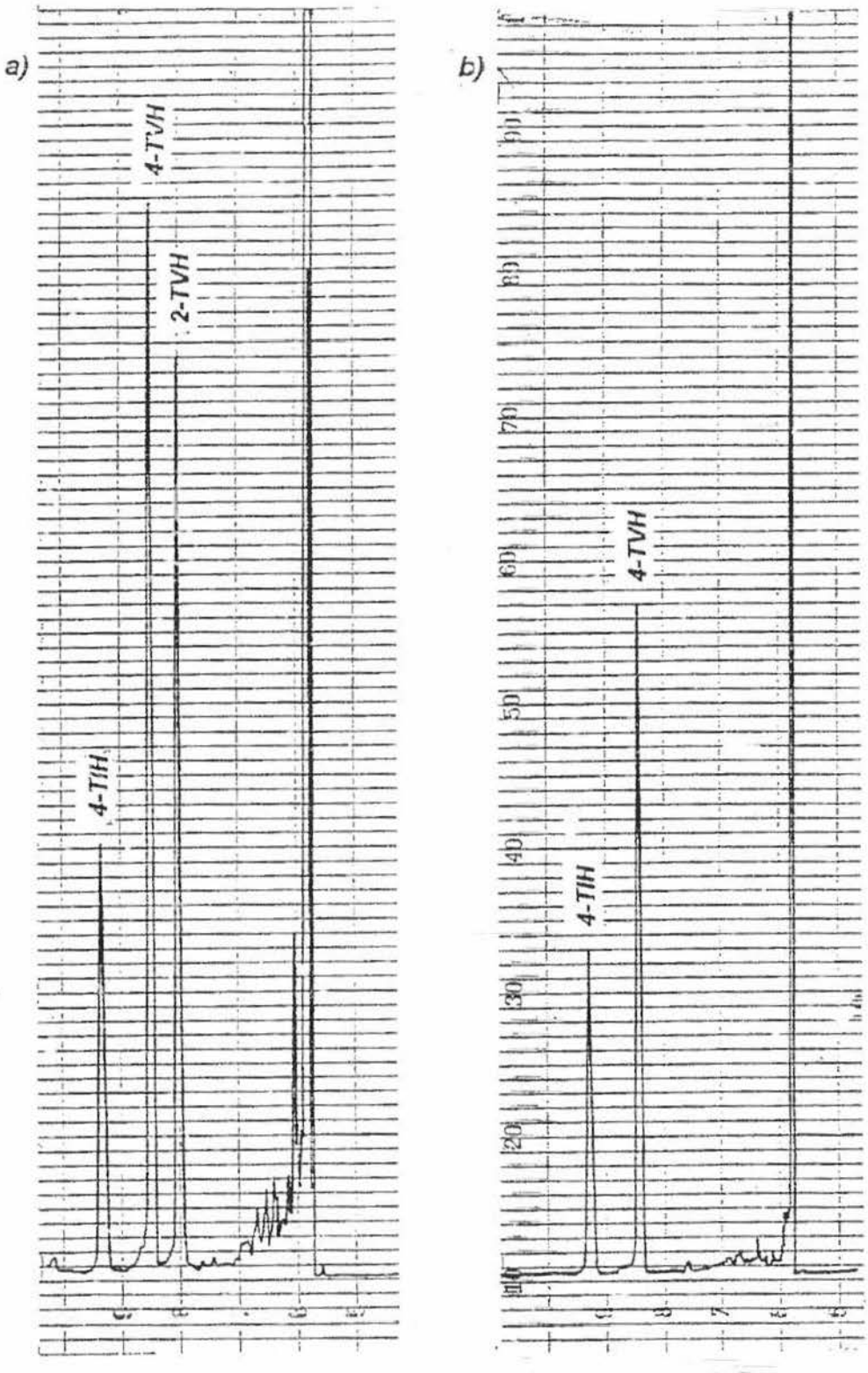
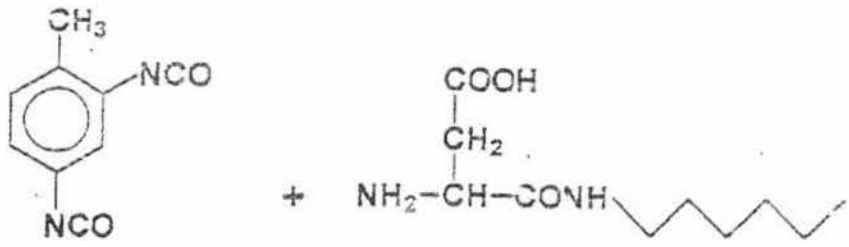
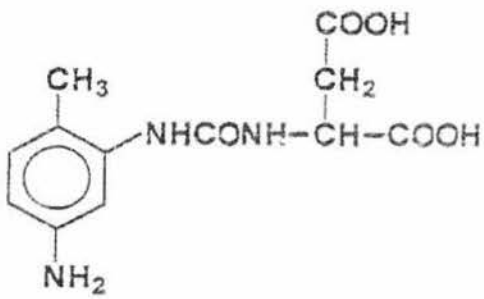



**Fig. 3 Gas chromatographic analysis of globins isolated from blood after incubation with 2,4-TDI (a) and 4-TMA (b)** [Globins $HG_{2,4\text{-TDI}(10)}$ (sample 6) and $HG_{4\text{-TMA}(1)}$ (sample 7B)]. 3-[4-(2-amino)tolyl]-5-isobutyl-hydantoin (4-TIH) was used as an internal standard. Chromatographic conditions: column 15m x 0.53mm x 1μm HP-50+; oven temperature 260°C; nitrogen-selective detector

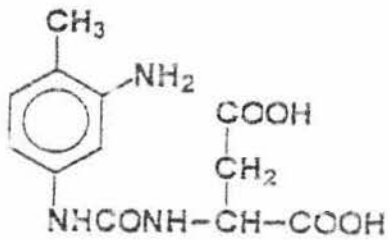


2,4-TDI

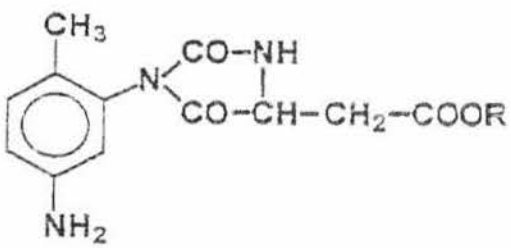
N-terminal Asp of albumin



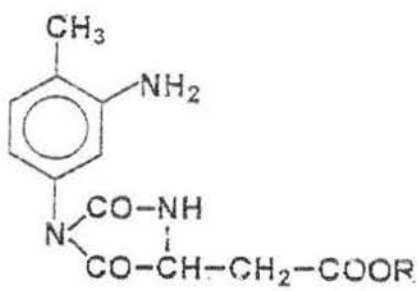
2-TAU



4-TAU

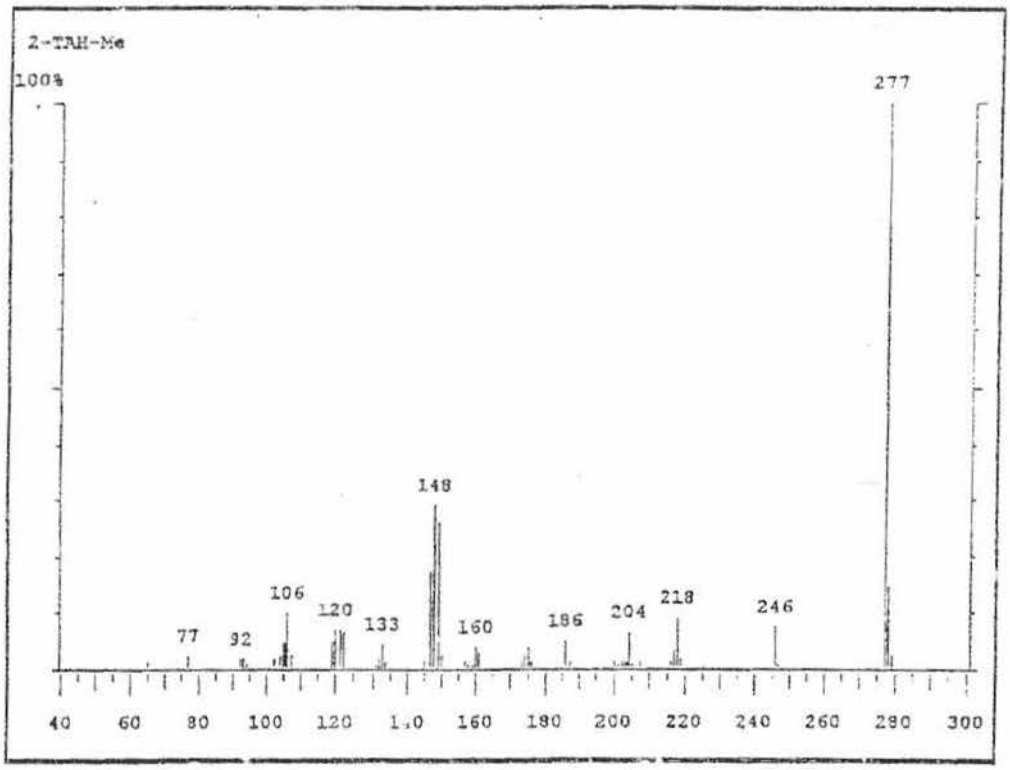


R = H: 2-TAH
$CH_3$: 2-TAH-Me



R = H: 4-TAH
$CH_3$: 4-TAH-Me

Fig. 4 Adducts of 2,4-TDI with N-terminal aspartic acid



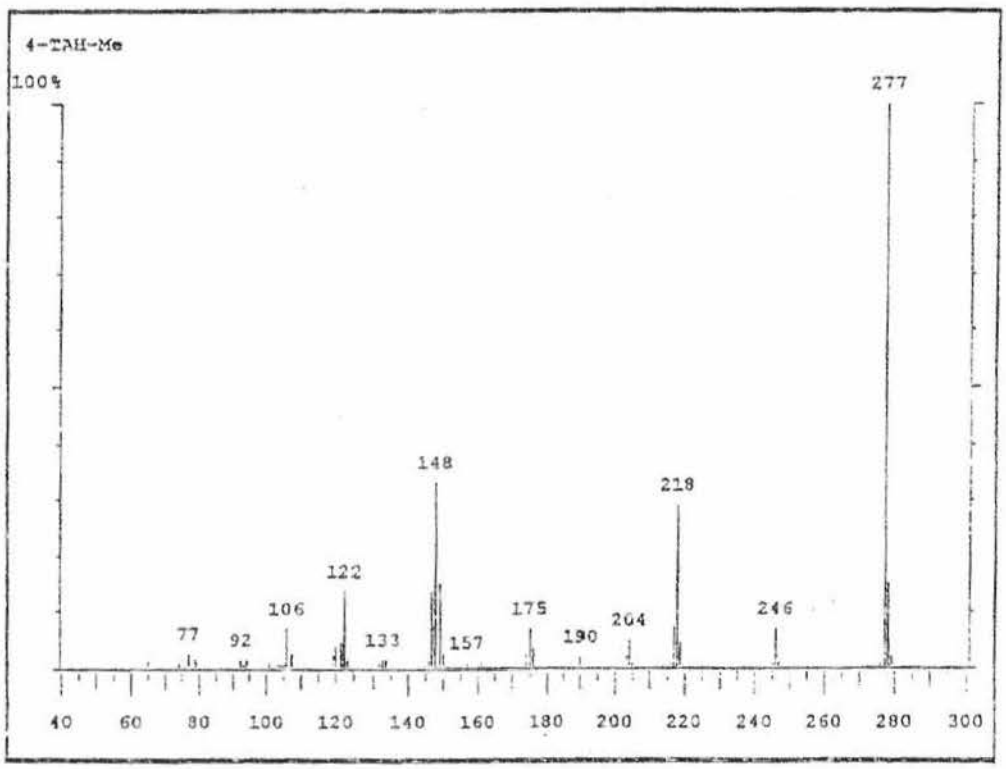




Fig. 5 Mass spectra of 2-TAH-Me and 4-TAH-Me

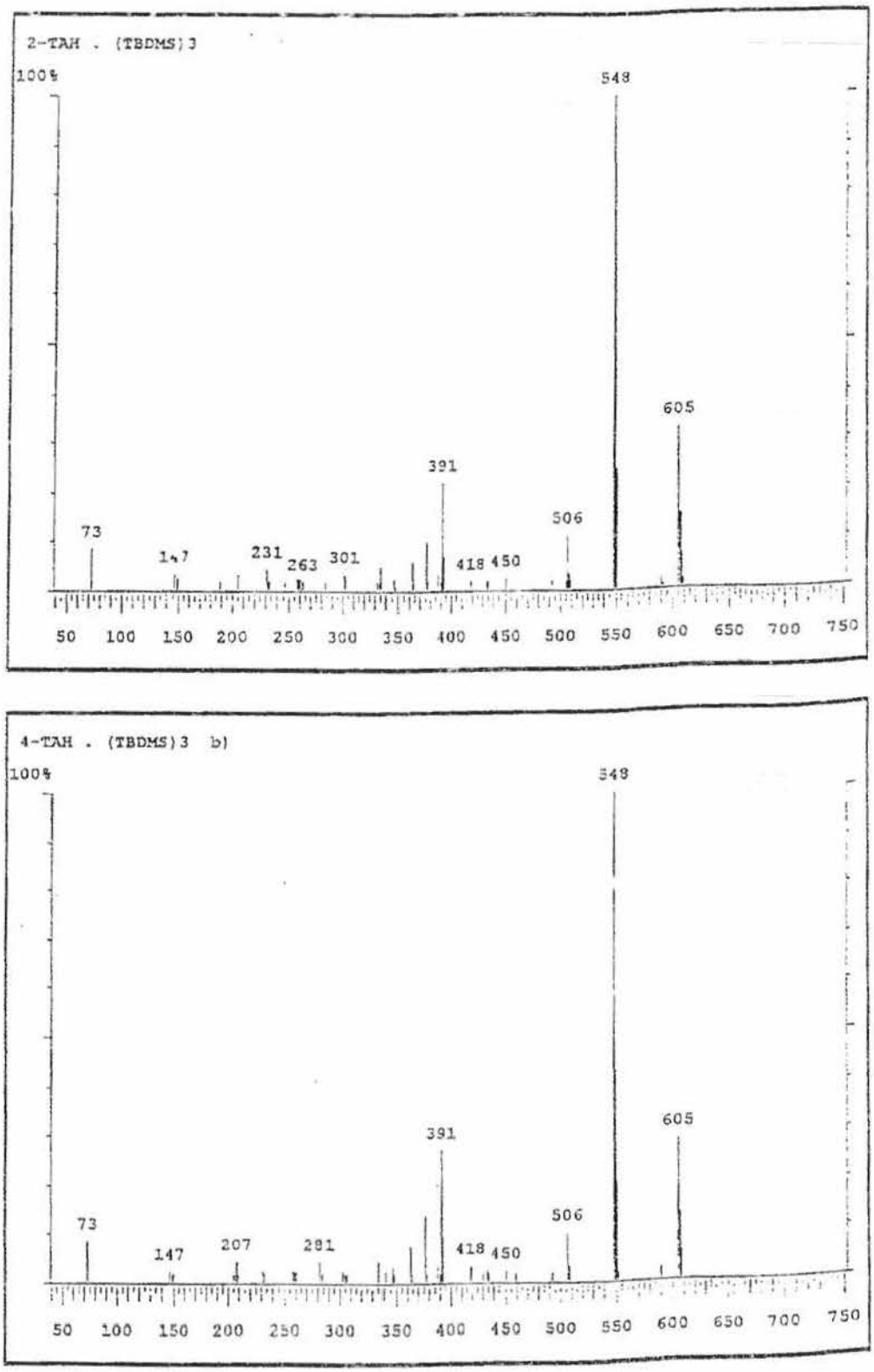



Fig. 6 Mass spectra of TBDMS derivatives of 2-TAH (Fig.7a, peak 2) and 4-TAH (Fig.7b, peak 5)

**Fig.7 GC-MS analysis of BSA incubated with 2,4-TDI and 4-TMA**

a) **2-TAH, TBDMS derivatives** (total ion chromatogram) [peak 1, $M^+$=719, 2-TAH-$(TBDMS)_4$; peak 2, $M^+$=605, 2-TAH-$(TBDMS)_3$]

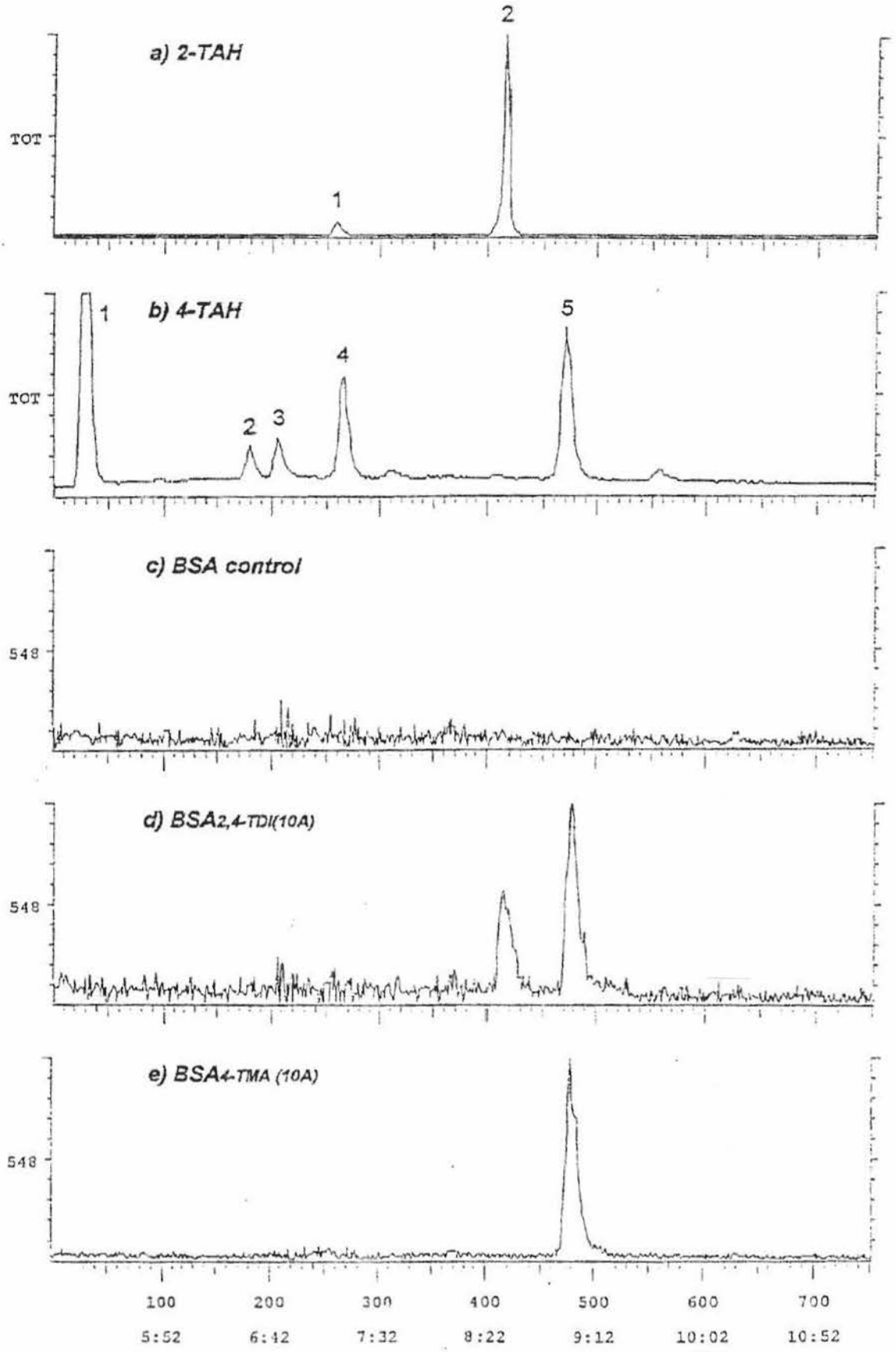
b) **4-TAH, TBDMS derivatives** (total ion chromatogram) [peak 1, $M^+$=605, 4-TAH-$(TBDMS)_3$; peak 2, $M^+$=498, 4-TAH-$(TBDMS)_2$; peak 4, $M^+$=719, 4-TAH-$(TBDMS)_4$; peak 5, $M^+$=605, 4-TAH-$(TBDMS)_3$]

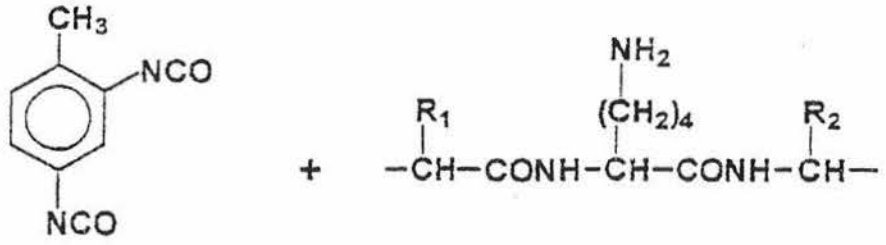
c) **BSA control, TAH-TBDMS derivatives** (m/z=548)

d) **BSA2,4-TDI(10A), TAH-TBDMS derivatives** (m/z=548)

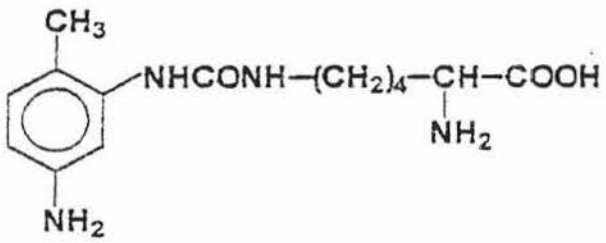
e) **BSA4-TMA(10A), TAH-TBDMS derivatives** (m/z=548)

Chromatographic conditions: column 30m x 0.25mm x 0.25µm DB-5ms; oven temperature 300°C; injector: split 1:20, injected 1µl

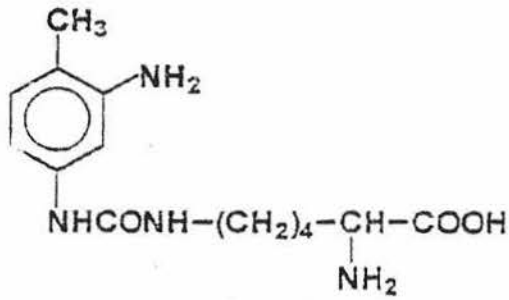

a) 2-TAH
TOT
1
2
b) 4-TAH
TOT
1
2
3
4
5
c) BSA control
548
d) BSA2,4-TDI(10A)
548
e) BSA4-TMA (10A)
548
100
200
300
400
500
600
700
5:52
6:42
7:32
8:22
9:12
10:02
10:52



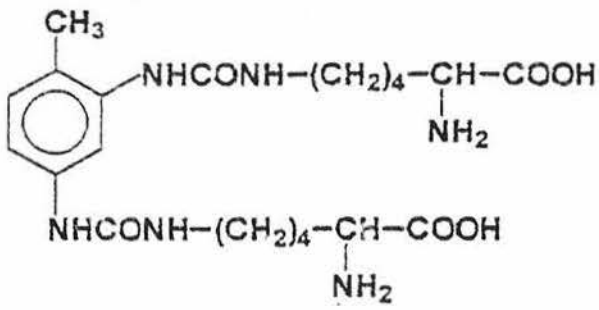

*2,4-TDI* *Lys*




*2-TLU*




*4-TLU*




*2,4-TDLU*

Fig. 8 Adducts of 2,4-TDI with lysine

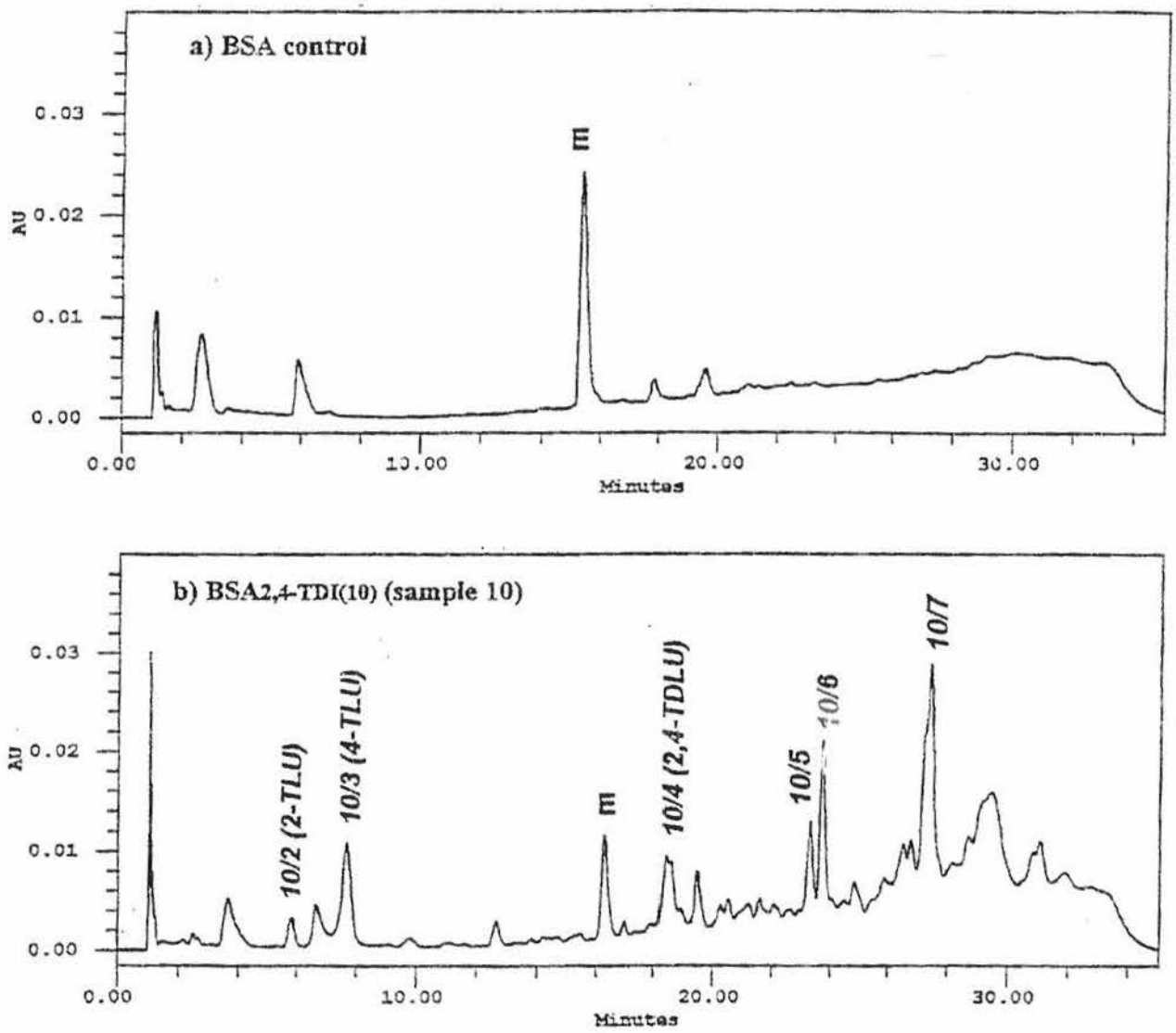



**Fig. 9 HPLC chromatogram of the enzymatic hydrolysate of a) BSA control, b) BSA2,4-TDI(10) (sample 10)**

Chromatographic conditions: column 150x3.9 mm NovaPak C18 (Waters); mobile phase MeOH (5-40%)-TFA(0.1%); detection: UV 240 nm

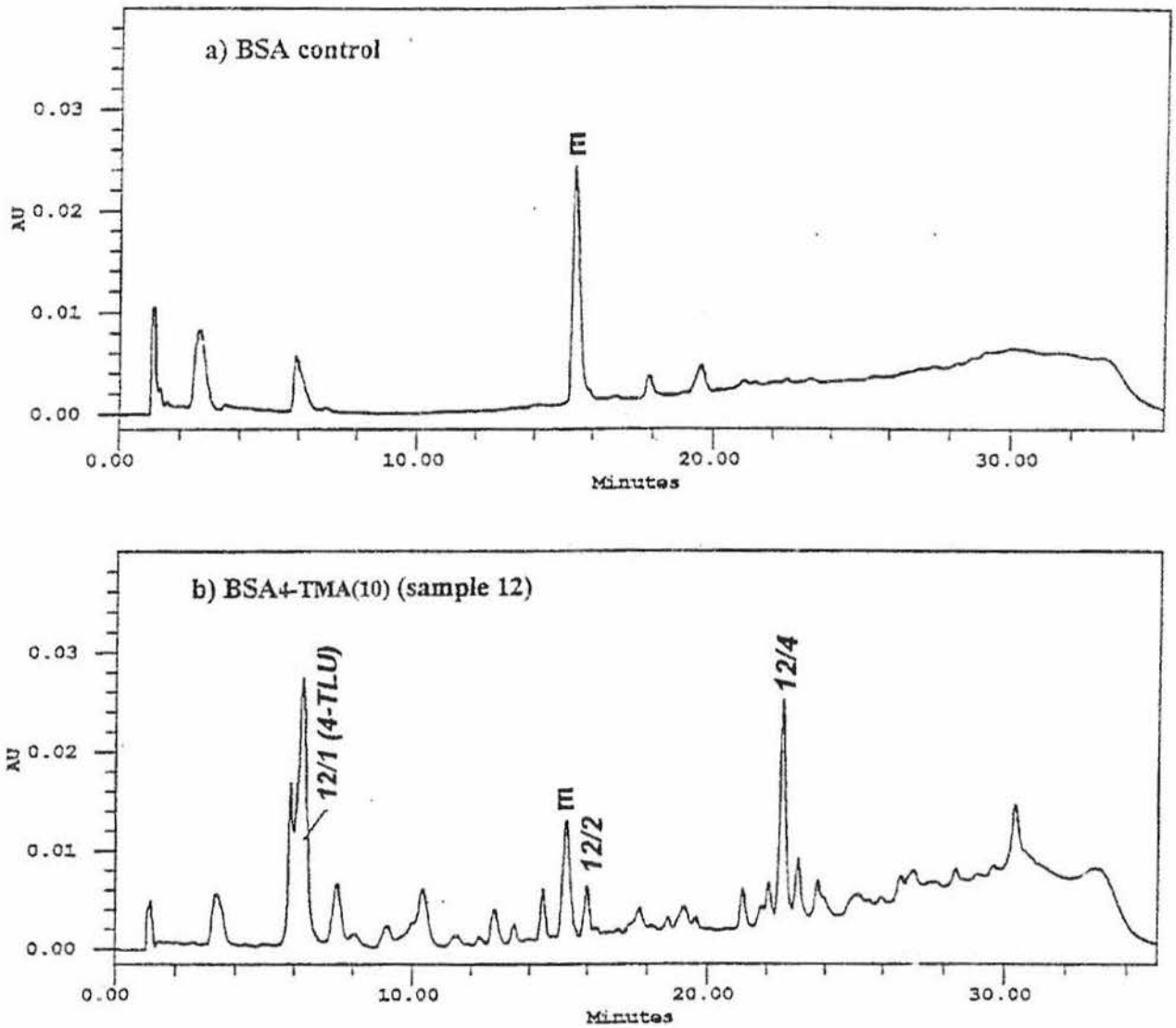



**Fig. 10 HPLC chromatogram of the enzymatic hydrolysate of a) BSA control, b) BSA4-TMA(10) (sample 12)**
Chromatographic conditions: column 150x3.9 mm NovaPak C18 (Waters); mobile phase MeOH (5-40%)-TFA(0.1%); detection: UV 240 nm

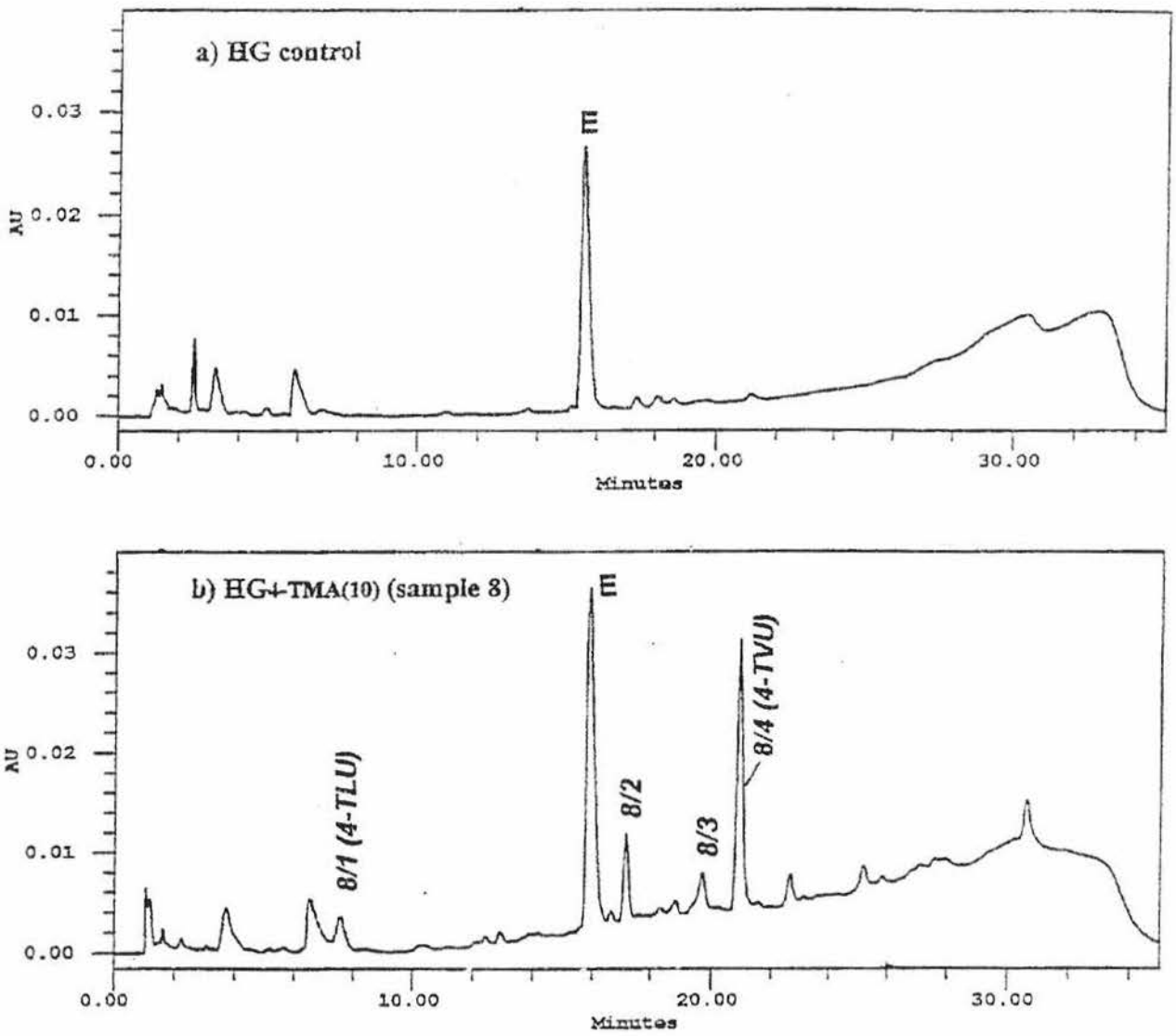
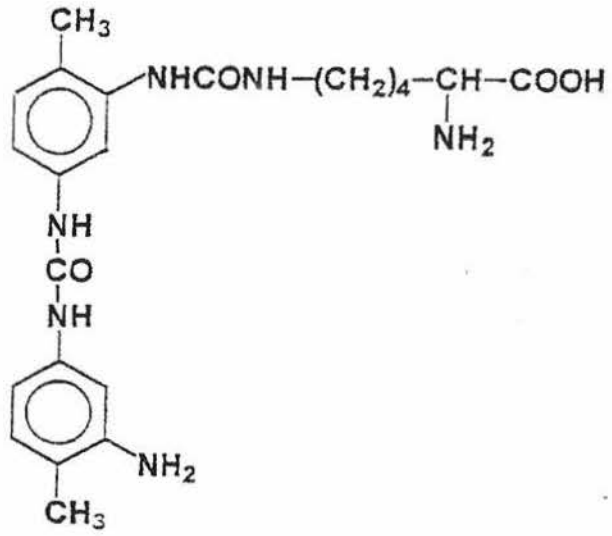
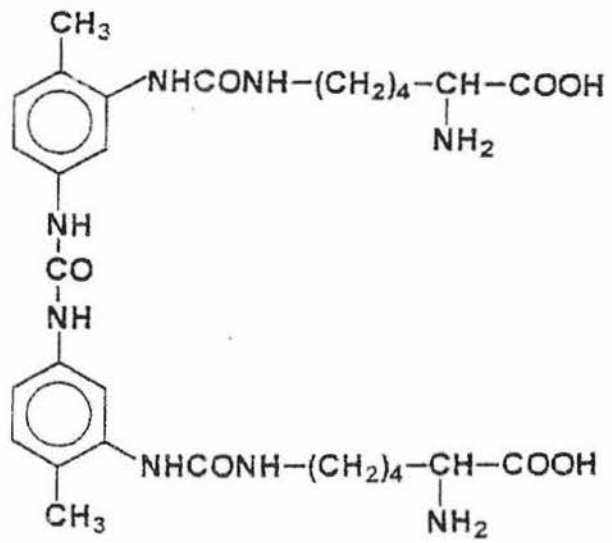



**Fig. 11 HPLC chromatogram of the enzymatic hydrolysate of a) HG control, b) HG+TMA(10A) (sample 8)**
Chromatographic conditions: column 150x3.9 mm NovaPak C18 (Waters); mobile phase MeOH (5-40%)-TFA(0.1%); detection: UV 240 nm




*MW=442*




*MW=614*

Fig. 13 Putative structures of the adducts with MW=442 and 614

C 10

## CERTIFICATE OF AUTHENTICITY

THIS IS TO CERTIFY that the microimages appearing on this microfiche are accurate and complete reproductions of the records of U.S. Environmental Protection Agency documents as delivered in the regular course of business for microfilming.

Data produced 7 (Month) 9 (Day) 99 (Year) Jia French Camera Operator

Place Syracuse (City) New York (State)

AMTEK corp